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Chapter 1: Introduction

Biophysical information from the cellular microenvironment such as extracellular matrix rigidity or mechanical forces, triggers intracellular signalling which results in various cellular functions such as proliferation, differentiation, migration and apoptosis through a process termed as mechanotransduction. The ability of cells to sense and respond to mechanical stimuli is essential for several developmental processes and postnatal homeostasis (Morin-Kensicki et al., 2006; Tian et al., 2007; Makita et al., 2008).

The stiffness of the substrate can regulate mesenchymal stem cells (MSCs) differentiation, with the stiffest substrate leading to osteogenic differentiation while soft substrate causing adipogenic differentiation (Engler et al., 2006; Dupont et al., 2011). In hESCs, substrate stiffness in combination with soluble molecules help maintain self-renewal on stiff substrate and caused neurogenic differentiation on soft substrate (Maldonado et al., 2015). Similar studies have reported that hPSCs on soft substrate differentiate into neuroectoderm (Hindley et al., 2016). However, no study has shown the interaction between hPSCs and substrate stiffness in absence of differentiation inducing medium. Understanding the interaction between hPSCs and stiffness is important because hPSCs are being used in many stem-cell based therapy especially in the light of new technologies such as 3D bio printing or tissue engineering biomaterials. Also, by controlling the stiffness of the substrates we may improve the regulation of the stem cell fate in bioartificial systems. Yes-associated protein (YAP) is a key mechanosensor that relays the mechanical signals into the nucleus. YAP is a transcriptional coactivator of the evolutionary conserved Hippo signalling pathway, which is an important regulator of organ size, cell proliferation and apoptosis (Wu et al. 2003; Huang et al. 2005; Dong et al. 2007).

Our aim was to study the effect of varying substrate stiffness on hPSCs differentiation into endoderm lineage.

Chapter 2: Review of Literature

Pluripotent stem cells (PSCs) are cells that have the capacity to self-renew and to develop into the three primary germ cells of the early embryo but not extra embryonic tissues such as the placenta. Embryonic stem cells (ESCs) are pluripotent cells, isolated from the inner cell mass of pre-implantation blastocyst, which can grow indefinitely and differentiate into any specialized cell of the body (Evans and Kauffman, 1981). Induced pluripotent stem cells (iPSCs) too are pluripotent stem cells but are generated by reprogramming of the adult stem cells using factors such as Oct3/4, Sox2, c-Myc and Klf4. They have properties similar to embryonic stem cells; therefore, can self-renew and differentiate into all cell types of the body (Takahashi & Yamanaka, 2006).

2.1 Mechanobiology

The microenvironment in which the stem cells reside consists of chemical, biological and mechanical signals which have a great influence on their survival, proliferation and differentiation. Along with signalling molecules, biophysical cues generated from extracellular matrix (ECM) stiffness, cell morphology or substrate topology plays an important role in regulating the signalling pathways in cells (Pelham & Wang, 1997; Li *et al.,* 2011). The cells sense these biochemical and mechanical stimuli generated from the extracellular environment by means of mechanical sensors present around the cell surface and transduced into the nucleus by mechanotransducers. This process is known as mechanotransduction. Integrins, cadherins and stretch-activated ion channels such as transient receptor potential channels (TRP channels) have been implicated as mechano-receptors which sense the signal from the extracellular environment. They relay the signals to the signal transducing molecules such as actin binding proteins, focal adhesion kinases, β-catenin, talin, Src, members of MAPK family and Rho family GTPases present in the cytoplasm. The signal is then transduced into the nucleus leading to target-specific gene expression (Holle *et al.,* 2013; Liu & Lee, 2014).

The effects of the substrate stiffness on the cellular behaviour have been studied extensively. Pelham and Wang (1997) showed that the kidney epithelial and fibroblast cells cultured on polyacrylamide gel respond to the difference in the substrate flexibility by altering their adhesion structures and motile behaviour. Furthermore, Engler *et al.* (2004a,

2006) reported that MSCs cultured on substrates of varying stiffness commit to phenotype corresponding to their biological tissue. Soft stiffness that mimics brain induces cells to commit towards neurogenic lineage, stiffer stiffness that mimic muscle induces cells to commit towards myogenic lineage, and comparatively rigid stiffness that mimic bone commit towards osteogenic lineage. Adding to this, Evans *et al*. (2009) demonstrated that the cell spreading, growth rate, gene expression and differentiation of ESCs is influenced by the change in the substrate stiffness. Many research groups have studied the importance of substrate stiffness in cell behaviour. Transcriptional co-activators: Yes-associated proteins (YAP) has emerged as mechanosensor which respond to the substrate stiffness and cell morphology (Dupont *et al.,* 2011; Aragona *et al*., 2013; Brusatin *et al.,* 2018; Lee *et al*., 2019).

2.2 YAP – Yes associated protein

YAP plays an important role in regulating organ size, normal tissue regeneration and also as potential therapeutic targets in cancer. They are the primary effectors of the Hippo pathway and are known to interact with TGFβ signalling pathway, WNT pathway, biophysical pathways and several others (Morgan *et al.,* 2013). The two kinases: MST and LATS, of the Hippo pathway regulate the localization of YAP. Phosphorylation of these two kinases by the upstream signals, causes phosphorylation of YAP, causing pYAP to be sequestered into the cytoplasm by 14-3-3 protein, and thereby degraded by proteosomes (Low *et al.,* 2014). In absence of the upstream signals, MST and LATS are unphosphorylated, causing YAP to translocate into the nucleus and bind to the DNA-binding transcription factors, such as TEADs, RUNX2, p-related p73 and the ErbB4 cytoplasmic domain, to activate the expression of certain genes (Lei *et al.,* 2008; Hong *et al.,* 2005).

In mouse ESCs, YAP is highly expressed under normal culturing conditions (Ramalho-Santos *et al.,* 2002). Previous studies demonstrated that YAP promotes stem cell self-renewal and pluripotency, and that loss of YAP leads to the loss of pluripotency in human and mouse ESCs (mESCs) (Lian *et al.,* 2010; Estaras *et al.,* 2017; Papaspyropoulos *et al.,* 2018). In addition, it was reported that YAP prevents hESCs differentiation and YAP overexpression suppresses mESCs differentiation (Lian *et al.,* 2010). Conversely, another study reported that YAP is dispensable for self-renewal, depletion of YAP inhibits

differentiation, and overexpression of YAP stimulates differentiation in mESCs (Chung *et al.,* 2016). It has also been shown that YAP depletion does not affect any of the normal stem cell characteristics in human induced pluripotent stem cells (hiPSCs) (Lorthongpanich *et al.,* 2020). These results suggest that the function of YAP is context specific and its role during human pluripotent stem cell differentiation needs has not been uncovered.

2.3 YAP as Mechanotransducer in stem cells

Dupont *et al.* (2011) identified YAP and TAZ as nuclear transducers of mechanical signals exerted by the ECM rigidity and cell shape. They reported that stiff substrate, large adhesive areas and in cells with high contractile forces activates YAP and promotes proliferation of primary mammary epithelial cells (MECs) and promotes MSCs differentiation towards osteogenic lineage. Conversely, YAP is inactive and in cytoplasm on soft substrate, small adhesive area and in cells with low contractile forces, causing apoptosis of MECs and differentiation towards adipogenic lineage of MSCs. Piccolo's laboratory reported that the subcellular localization and activity of YAP is regulated by actin cytoskeleton remodelling, cell substrate rigidity and topography, and cell stretching. The stiff substrate and high filamentous actin (F actin) levels have been shown to result in their nuclear translocation (Aragona *et al.,* 2013). Thus, confirming that substrate stiffness regulates the YAP activity in MSCs and hPSCs.

However, effect of substrate stiffness on human pluripotent stem cells (hPSCs) is not clearly understood. hESCs cultured on varying substrate stiffness, in presence of soluble pluripotency factors, show YAP nuclear localization and differentiation towards post mitotic neurons (Musah *et al.,* 2014). While one study states that hPSCs maintained the stemness on stiff substrates and undergo neurogenic differentiation of soft substrates (Maldonado *et al.,* 2015). Another study reports that neural induction is initially enhanced on soft substrate, but differentiation into neural progenitors and motor neurons occurs on stiff substrate. Additionally, mesoendodermal differentiation enhances on stiff substrate but further specification to posterior foregut requires soft substrate (Maldonado *et al.,* 2017).

Smith *et al.* (2017) highlighted that by changing the stiffness of the polydimethylsiloxane (PDMS) substrate the mesoderm differentiation kinetics of hiPSCs can be modulated and this mechanical change activates YAP during mesoderm induction. The

main aim was to achieve endothelial commitment of the hiPSCs when cultured on substrates of varying stiffness. These contrary results in hPSCs indicates that dynamic changes of substrate stiffness effects differentiations.

Chapter 3: Lacunae/Rationale, Aim and Objectives

3.1 Lacunae/Rationale

Growth factors and chemical cues are well known regulators of stem cells, but recent studies demonstrated the role of biophysical signals in regulating stem cell proliferation and differentiation through mechanotransduction pathways. Many research groups have reported the effect of artificial substrate on stem cells by varying its stiffness. Additionally, Hippo signalling pathway and its downstream effectors YAP have been shown to synchronize various signalling pathways and physical interactions between cells and its surrounding environment to bring about change in gene expression (Dupont *et al.*, 2011). YAP and TAZ are also known to play a crucial role in maintaining the "stemness" of the mouse pluripotent stem cells (Lian *et al.*, 2010), however, whether YAP regulates lineage specification in human pluripotent stem cell differentiation has not been demonstrated.

The studies on YAP and TAZ have focused on the fate of MSCs on different substrates and the role played by YAP during this process (Engler *et al.*, 2006; Dupont *et al.*, 2011; Smith *et al.*, 2017). Few studies that have used human pluripotent stem cells have uncovered role of YAP activity in maintaining the undifferentiated state and their inhibition led to neuronal differentiation. There are several unknowns that need to be studied with respect to YAP activity in human pluripotent stem cells such as: expression levels and localization of YAP in undifferentiated and differentiated cells, activity of YAP in germ lineages other than ectoderm, expression of YAP in embryoid bodies in suspension culture which has been shown to promote ectoderm differentiation, how does hPSCs differentiate on stiff matrix and how is the activity of YAP affected during the process, does inhibition of YAP activity affect pluripotency or differentiation capacity of stem cells, how antagonistic signalling pathways affect YAP activity and the subsequent effect on fate of stem cells.

Our primary aim was to investigate the interaction between hPSCs and substrate stiffness during their differentiation. And to understand if this interaction has any effect on the localization of YAP and TAZ. The study focuses on the YAP expression during the differentiation of hPSCs into endoderm lineage in response to substrate stiffness. We have modulated the levels of YAP by changing substrate stiffness and investigated if the altered

levels of YAP have any effect on the cell fate. These basic interactions will help us understand whether YAP is the integral part of hPSCs differentiation or not.

3.2 Aim of the project

To study the activity of YAP and TAZ during differentiation of human pluripotent stem cells (hPSCs) into endoderm lineages in response to different substrate stiffness.

3.3 Objectives

1. Investigate the expression of phosphorylated and non-phosphorylated YAP/TAZ in undifferentiated human pluripotent stem cells on substrates of different stiffness
2. Expression of phosphorylated and non-phosphorylated YAPZ/TAZ in hPSCs during endoderm differentiation on the substrates of different stiffness.
3. Effect of pharmacological inhibitor/activator of YAP/TAZ on hPSCs during endoderm lineage differentiation on substrates of different stiffness.

Chapter 4: Materials and Methods

4.1 Ethical statement

hESC cell line KIND1 (Kumar *et al.*, 2009) was procured from National Institute for Research in Reproductive Health (NIRRH), Mumbai, India; the use of these cell line was approved by the Institutional Committee for Stem Cell Research (ICSCR), NMIMS (deemed-to-be) University, Mumbai, India. Human placental mesenchymal stem cells (hPMSCs) were procured from Symbiosis Centre for Stem Cell Research (SCSCR). The cell line was used in NCCS & SCSCR and approved by the IC SCR committee of NCCS.

4.2 Substrates used in the study

For positive control, traditional polystyrene tissue culture treated plastic dishes (TCP) (Corning, USA) were used. For softer substrates, commercially available CytoSoft® plates (Advanced BioMatrix, USA) were used. These plates have polydimethylsiloxane (PDMS) layer and elastic modulus of 0.2kPa , 0.5kPa, 2kPa, 8kPa, 16kPa, 32kPa, to 64kPa. The Cytosoft® (CS) substrates were activated as per manufacturer's instructions. Briefly, each well was coated with3mL of 1X vitronectin (Thermo Fisher Scientific, Life technologies, CA, USA) for 1 hour at 37˚C, followed by three washes with 1x DPBS for 5 mins and cell seeding. The 96-well ultra-low attachment dishes (ULAD) (Corning, USA) was also used which gave us the near-suspension culture conditions. In TCP and CS cells were cultured for 4-5 days on each substrate before passaging and in ULAD cells were cultured for 7 days. KIND1 cells were maintained for three consecutive passages on all the substrate.

4.3 Maintenance of hESCs

hESCs, KIND1 cells, were cultured and maintained in xeno- and feeder-free system using 1X vitronectin. All the substrates i.e., TCP and CS were coated with 1X vitronectin. hESCs were maintained in pluripotency maintaining medium (PMM), Essential 8 basal medium (E8; Thermo Fisher Scientific, Gibco) supplemented with 1X Essential 8 supplement (Life Technologies) and incubated at 37˚C, 5% CO_2. The media was changed daily. When the cells attained 80% - 90% confluency, they were passaged using 0.5mM EDTA (Sigma Aldrich, MO, USA) at a passage ratio of 1:4. In ULAD, 200µl of the cell suspension was added to each well

and incubated at 37˚C, 5% CO_2. The cells were cryopreserved in cryoprotectant medium containing E8 and 10% DMSO (Sigma Aldrich) and stored in liquid nitrogen.

4.4 Differentiation of hESCs with minimum differentiation inducing media

To study whether substrate stiffness alone can induce differentiation of hESCs, we cultured hESCs in differentiation inducing media (DIM) with minimum serum concentration. KIND1 cells were maintained in Advanced Dulbecco's Modified Eagle Medium (DMEM) (Thermo Fischer Scientific, USA) which was supplemented with 2% foetal bovine serum (FBS) (Thermo Fischer Scientific, USA) and 1% antibiotic, without any lineage specific growth factors for 4 days.

4.5 Directed differentiation towards definitive endoderm

Undifferentiated KIND1 cells showing 80% - 85% confluency were used for differentiation and RNA was extracted on day 4 of the differentiation protocol. Briefly, cells were rinsed with DPBS followed by culturing in RPMI-1640 (Sigma-Aldrich) media supplemented only with 0.025× ITS (Sigma-Aldrich) and 100 ng/ml ACTIVIN A (R&D Systems) on the first day and an additional three days with increasing concentration of foetal bovine serum from 0.2%, 0.5% and 2% on days 2, 3 and 4 respectively. KIND1 differentiated on TCP were used as control for all the studies.

4.6 Inhibitor and Activator

For modulation study, KIND1 cells were differentiated on TCP, and CS plates of stiffness 0.2kPa and 64kPa. 30nM of YAP inhibitor, Verteporfin (Sigma-Aldrich), and 10uM of YAP activator, Lysophosphatidic acid (Sigma-Aldrich) was added to the differentiated cells on day 4 of the differentiation protocol. Equal volume of DMSO was added to each substrate as vehicle control. The cells were collected for RNA and protein post 24 hours after treatment.

4.7 Maintenance of hMSCs

For hPMSCs, TCP and CS substrates were coated with 1X collagen. hPMSCs were maintained on TCP and CS substrates in Improved Minimum Essential Medium (IMEM) (Thermo Fischer Scientific, USA) medium supplemented with 10% FBS and antibiotics and maintained them in culture for four days.

4.8 RNA extraction and Quantitative Reverse Transcription RCR

Total RNA was isolated using TRI reagent (Sigma Aldrich, USA), and was quantified using BioTek™ Epoch™ Microplate Spectrometer (BioTek, USA). Complementary DNA (cDNA) was prepared from 1µg of total RNA using Prime Script 1st Strand cDNA synthesis kit (TakaraBio, Japan) as per the manufacturer's recommendations in Nexus Thermal Cycler (Eppendorf, Germany). The quantitative real time PCR was performed in QuantStudio 3 and Thermal cycler (Applied Biosystems, CA, USA). For each sample, the relative fold change was calculated by $2^{-\Delta\Delta Ct}$ method. Each PCR reaction was carried out in duplicates using samples from three biological replicates. Endoderm differentiated day 4 (ED D4) cDNA was used as positive control for endoderm and mesoderm; and commercially available adult human brain RNA sample (Takara Bio) was used as positive control for ectoderm. The following primers were designed using NCBI Primer Blast and used in the study: Pluripotency: *OCT4, NANOG* and *SOX2*; Lineage specific primers: Endoderm specific – *SOX17*, *FOXA2*, *CXCR4*; Mesoderm specific – *BRACHYURY*; Ectoderm specific – *PAX6*; Housekeeping: *18S rRNA*

4.9 Protein extraction and Western blot

Proteins were extracted by resuspending the cell pellet (obtained after EDTA treatment) in chilled complete cell lysis buffer containing 1X Protease inhibitor cocktail (Sigma Aldrich) and Roche PhosSTOP (Sigma Aldrich). The total protein concentration was by the Lowry assay using Folin–Ciocalteu's phenol reagent (Sigma Aldrich). 20ug-50ug of the total protein was loaded on 10% SDS-PAGE. For transfer, PVDF membrane (Biorad, USA) was used. The primary antibodies used were: anti-OCT4A, anti-Cyclin D1, Hippo signalling pathway kit (CST, USA); anti-YAP (Sigma Aldrich); anti-NANOG (Abcam, USA); and loading control anti-GAPDH (CST, USA) followed by detection with the anti-rabbit HRP linked secondary antibody (CST).

4.10 Immunofluorescence

KIND1 cells cultured on glass coverslips in pluripotency maintaining medium (control, TCP) and KIND1 cells cultured in ULAD were transferred to glass coverslips and allowed to expand for 24 hours, before fixing them with 4% paraformaldehyde. The primary antibody used for immunofluorescence are: anti-OCT4A and anti-YAP XP® (Cell Signalling Technology). The secondary antibody Anti-Rabbit IgG (H+L) Alexa Fluor 488 (Thermo Fisher) was used.

Chapter 5: Investigate the expression of phosphorylated and non-phosphorylated YAP/TAZ in undifferentiated human pluripotent stem cells on substrates of different stiffness

We have cultured hESCs on TCP, CS substrates and ULAD in pluripotency maintaining media. TCP was used as control, CS substrate provided a range of stiffness: 0.2kPa, 0.5kPa, 5kPa, 8kPa, 16kP, 32kPa and 64kPa, and ULAD gives us no-substrate system. On soft substrate, hESCs have been reported to under matrix-induced differentiation towards neurogenic lineage. We wished to see how hESCs respond under stress.

5.1 Characterization of hESCs cultured on TCP

KIND1 cells cultured in E8 media on TCP, were first characterized for pluripotency by end point PCR and confirmed by immunofluorescence. Expression of pluripotency markers *OCT4, NANOG* and *SOX2* were seen and no expression of lineage specific markers *SOX17*, *BRACHYURY* and *PAX6* was observed. Moreover, localization of OCT4 protein in the cell nuclei confirmed that cells were in the undifferentiated state.

5.2 Maintaining hESCs on CS substrates in Pluripotency maintaining media (PMM)

To identify whether the biophysical cues alone can induce differentiation in pluripotency supporting media, we cultured hESCs on tissue culture treated plastic (TCP) dishes and on CytoSoft® (CS) substrate plates in pluripotency maintaining for three consecutive passages. The cells were collected for RNA and protein at day 4 of each passage. In pluripotency maintaining medium hESCs on TCP were present in compact colonies with characteristic epithelial morphology. Surprisingly, hESCs cultured on CS substrates exhibited similar morphology and cells were arranged in compact colonies. We characterized the hESCs cultured on TCP and CS substrates for OCT4 and NANOG protein expression. Compared to their expression on TCP, OCT4 and NANOG levels were maintained in all the three consecutive passages. To confirm whether lineage differentiation had occurred, we checked the mRNA expression of lineage specific markers - *SOX17* (endoderm), *BRACHYURY* (mesoderm) and *PAX6* (ectoderm). No expression of lineage specific markers was observed by real time PCR. Our results showed that hESCs remained pluripotent on soft substrates in presence of pluripotency maintaining factors.

5.2.1 Correlation between YAP and substrate stiffness

In hESCs, YAP has shown to maintain pluripotency on TCP and overexpression of YAP has been correlated to naive pluripotency (Qin *et al*., 2016). So, we wondered whether YAP plays any role in maintaining the pluripotent state of hESCs on soft substrates as well. Protein samples isolated from TCP and CS substrates were probed with YAP and pYAP antibodies. The western blot data showed almost equivalent expression of YAP and pYAP on all the substrates. KIND cells were cultured on glass coverslips (stiff substrate) and probed with YAP antibody. We saw the expression of nuclear YAP in the nucleus and cytoplasm corelating with the western blot data. Summarizing the above results, we can say that contrary to the earlier reports, we did not see any change in pluripotency of hESCs despite culturing them on substrates with stiffness several thousand-fold lower than conventional plastic dishes.

5.2.2 Maintaining hESCs in ULAD in PMM

Since all our substrates retained stemness, we were intrigued to see whether culturing KIND1 in near suspension like conditions can affect stemness. We maintained KIND1 cells on ULAD for seven days in pluripotency maintaining media. We subsequently passaged hESCs till three passages. We observed almost equal expression of OCT4 protein in ULAD in all the three passages. OCT4 expression was confirmed by immunofluorescence staining. No expression of lineage specific markers seen., therefore, we can say that hESCs cultured without substrate retained their stemness. Significant upregulation of YAP was observed in ULAD compared to TCP. We can speculate that YAP might regulate pluripotency but this needs extensive evaluation. We did not carry this study further because our other systems are 2D and the cells on ULAD form tight clumps which are 3D. Therefore, comparing 2D and 3D results might give misleading results.

5.3 Maintaining hESCs on CS substrates in Differentiation inducing media (DIM)

The above results roused our curiosity, if change in substrate stiffness alone does not induce differentiation, then whether changing the medium to minimum differentiation inducing medium could lead to any specific lineage as reported previously with hMSCs. We intentionally kept the serum concentration to 2%, as we did not want the serum components to dictate the course of differentiation, but still added enough serum to keep

cells healthy. We switched the media to Advanced DMEM with 2% FBS and checked the expression of pluripotency markers, lineage specific markers, YAP and pYAP.

The hESCs colonies in presence of differentiation inducing medium lost their compact epithelial morphology and appeared flattened. As expected, the induction of differentiation was marked by downregulation of OCT4 expression in cells grown on TCP, however, surprisingly the cells grown on softer substrates showed relatively higher OCT4 expression. Next, we studied gene expression of lineage specific markers by quantitative reverse-transcription PCR to assess lineage specification. A comparative analysis between positive control and other samples showed reduced expression of *SOX17*, *BRACHYURY*, and *PAX6* in TCP and CS. These results hint that the hESCs on soft substrates in the presence of low levels of morphogens differentiates but not towards specific lineage.

5.3.1 Correlation between YAP and substrate stiffness

Next, we performed immunoblotting to check the dynamics of YAP and pYAP proteins in differentiation inducing media on soft substrates. The comparative study of YAP and pYAP showed low expression of YAP and relatively high expression of pYAP. Low expression of YAP has been reported to cause loss of stem cell pluripotency (Lian *et al*., 2010; Rosado-Olivieri *et al*., 2019) and pYAP gets sequestered in the cytoplasm leading to no YAP expression. Our results imply that on soft substrates and in presence of minimum differentiation inducing signals, hESCs start losing their pluripotency and undergo differentiation.

5.4 Pilot study of hPMSCs

Most of the substrate studies use MSCs, therefore, we wished to replicate the reported results with hPMSCs. On TCP, hPMSCs showed elongated fibroblast like morphology. hPMSCs cultured on 0.2kPa, 0.5kPa and 5kPa showed less spreading and cell count, however, hPMSCs cultured on 8kPa, 16kPa, 32kPa and 64kPa showed similar morphology to TCP. Just from the visual confirmation we can deduced that MSCs are sensitive to the change in stiffness compared to hESCs, which resisted the change in stiffness.

Chapter 6: Expression of phosphorylated and non-phosphorylated YAPZ/TAZ in hPSCs during endoderm differentiation on the substrates of different stiffness

To investigate the stiffness-induced changes during stem cells differentiation, we cultured hESCs substrates of different stiffness. KIND1 cells were differentiated towards definitive endoderm lineage on TCP and CS plates. After day 4 of the differentiation procedure, cells were harvested for RNA and protein. The hESCs on TCP and CS substrates showed transition from pluripotent cell morphology (compact colonies) to elongated, flattened morphology. Expression of pluripotency marker OCT4 at day 4 of endodermal differentiation was observed (Pethe *et al*., 2014; Dumasia *et al*., 2021), however upregulation of lineage specific gene markers *SOX17*, *FOXA2*, *CXCR4,* and *BRACHYURY* by reverse transcription PCR, indicated the differentiation of hESCs into mesendoderm lineage. No expression of ectoderm marker *PAX6* was observed.

Our next step was to assess the expression of YAP and pYAP in differentiating cells. The purpose of this study was to determine whether substrate stiffness as well as differentiation affects YAP expression. YAP is a critical component of Hippo signalling pathway which when unphosphorylated, relays mechanical signal into the nucleus thereby deciding cellular fate. pYAP expression normalised with relative YAP levels did not show significant change in the expression levels compared to the TCP. Whereas, YAP levels when normalised with the relative GAPDH levels showed slight downregulation compared to TCP. Our results suggests that substrate stiffness does not affect YAP levels, and the cells maintain total YAP expression during the differentiation.

Chapter 7: Effect of pharmacological inhibitor/activator of YAP/TAZ on hPSCs during endoderm lineage differentiation on substrates of different stiffness

In this chapter we discuss the results of modulation of YAP levels during differentiation on substrates of varying stiffness.

7.1 YAP inhibition by Verteporfin

Verteporfin (VP) has been used in to demonstrate the effect of YAP inhibition on stem cell regulation (Rosado-Olivieri *et al*., 2019; Dong *et al*., 2020; Quan *et al*., 2021). However, inhibition of YAP in hESCs cultured on varying stiffness has not been demonstrated. The exact mechanism of action of Verteporfin is unknown, however it has been reported that VP selectively binds to YAP causing some conformational changes in YAP structure, thereby eliminating YAP interaction with DNA binding proteins. Another mechanism is that, VP increases the levels of 14-3-3 protein in cytoplasm thereby blocking the YAP function (Wang *et al*., 2016). YAP inhibition has shown to enhance differentiation of hESCs (Rosado-Olivieri *et al*., 2019; Quan *et al*., 2021).

For our study we used a large range of VP concentration from 10nM, 20nM, 30nM, 40nM, 50nM, 60nM, 70nM and 80nM to standardize the VP concentration. hESCs were cultured on TCP in complete E8 medium and treated with the mentioned concentrations on day 4. DMSO as vehicle control was used. We observed that hESCs treated with concentration above 30nM showed abnormal cell death. The cells from remaining three concentrations were harvested for protein, and YAP expression was observed. 30nM VP concentration showed significant downregulation in YAP expression compared to the DMSO control, hence this concentration was used for further studies.

Next, we went ahead to understand the effect of YAP inhibition on endoderm differentiation. KIND1 cells were cultured on TCP, CS substrates with stiffnesses 0.2kPa and 64kPa. KIND1 was differentiation was per above mentioned protocol and the differentiated cells were treated with 30nM of VP on day 4 of the differentiation. Next day, the cells were harvested for RNA And protein. To confirm the differentiation state mRNA levels of lineage specific markers were analysed. Expression of definitive endoderm markers: *SOX17, FOXA2*, and *CXCR4*, and mesoderm marker: BRACHYURY confirmed that the hESCs have undergone

differentiation into definitive endoderm stage. *SOX17* and *CXCR4* showed similar pattern throughout the various stiffness, with gradual decrease from TCP to 64kPA. High expression of *FOXA2* and *BRACHYURY* in 0.2kPa VP treated cells indicate that cells might favour soft substrate for mesoendoderm differentiation. To confirm whether YAP inhibition promoted differentiation we checked for the YAP proteins levels. YAP levels were downregulated in TCP which corelates to the literature. Surprisingly, we observed an upregulation of YAP in 0.2kPa and 64kPa compared to the TCP DMSO control. From the inhibition study, we can conclude that the substrate stiffness and the inhibitor have an effect on the differentiation of hESCs other than the one reported in the literature and this can be potentially explored further.

7.2 YAP activation by Lysophosphatidic acid

Intrigued by the inhibitor study, we added YAP activator, LPA during definitive endoderm. LPA is a small lipid molecule which inhibits Hippo pathways by dephosphorylating LATS, thereby activating YAP expression in cells (Yu *et al*., 2012). In 2016, Qin and colleagues found that activating YAP activity by supplementing lysophosphatidic acid (LPA) significantly induced the transition from the primed to the naïve state in multiple human ESC and iPSC lines, and the naïve state was prolonged in the culture medium supplemented with LPA. These results suggest an unexpected role of YAP in regulating the induction and maintenance of human naïve stem cells. For standaridizing LPA concentration, we treated undifferentiated cells cultured on TCP with 10µM, 15µM and 20µM concentration of LAP on day 4. The protein expression study showed that 10µM of LPA showed significant increase in YAP expression compared to respective DMSO control and hence we used this concentration for differentiation studies.

Next, we went ahead to understand the effect of YAP inhibition on endoderm differentiation in hESCs. KIND1 cells were cultured on TCP, CS substrates with stiffnesses 0.2kPa and 64kPa. KIND1 was differentiation was per above mentioned protocol and the differentiated cells were treated with 10uM of LPA on day 4 of the differentiation. Next day, the cells were harvested for RNA And protein. To confirm the definitive endoderm differentiation and also the effect of stiffness on the differentiation, mRNA levels of lineage specific markers were analysed. LPA treated cells showed upregulation of *SOX17, FOXA2,* and *CXCR4* confirming the definitive endoderm stage of differentiated cells. Additionally,

LPA treated cells seemed to favour differentiation compared to the respective DMSO control more in 0.2kPa stiffness compared to TCP and 64kPa stiffness. YAP protein expression was high on CS substrates compared to TCP DMSO control, however, no significant change in YAP expression was observed.

Chapter 8: Discussion

In the project, we intended to determine the role of substrate stiffness and YAP in human pluripotent stem cells during their undifferentiated and differentiated state. Our data showed that for hESCs the biochemical signals can keep cell in undifferentiated or differentiate them, but changes in substrate stiffness does not affect the hESCs. Differentiation of hESCs on TCP and soft substrates was observed after the addition of differentiation inducing media, as seen by the expression of selective lineage markers.

We shed a light on the connection between the biochemical and biophysical signals during endoderm differentiation of hESCs cultured on substrate of varying stiffnesses, which is still an active area of research. Several research groups have demonstrated that stem cells specifically hMSCs and mESCs respond to the changes in substrate stiffness and differentiate into neurogenic, myogenic or osteogenic lineage on substrate stiffness mimicking respective biological tissue stiffness (Engler *et al*., 2006; Evans *et al*., 2009; Keung *et al*., 2012), and that YAP is an important transcriptional regulator in maintaining pluripotency and stiffness-induced stem cell behaviour (Dupont *et al*., 2011; Oliver-De La Cruz *et al*., 2019; Musah *et al*., 2014; Brusatin *et al*., 2018; Lee *et al*., 2019). Under normal culture condition, downregulation of YAP in mESCs has shown to cause differentiation into endoderm and mesoderm lineages (Lian *et al*., 2010). *In vitro,* ectopic expression of YAP inhibits ESC differentiation and cells maintain stem cell phenotype under differentiation conditions. Subsequently, LIF (Leukaemia inducing factor) (Tamm *et al*., 2011) and Inter-α-Inhibitor (IαI), a component of serum (Pijuan-Galitó *et al*., 2014), were identified to facilitate YAP expression and induce OCT3/4 expression. A novel method showed that overexpression of two reprogramming factors OCT4 and SOX2 (Takahashi and Yamanaka 2006) and YAP, key component of Hippo pathway, together induced reprogramming of human amniotic epithelial cells into induced pluripotency stem cells (Zhao *et al*. 2017). However, there are no reports on how YAP regulates with pluripotency in human embryonic stem cells.

Intrigued by these studies, we asked whether culturing the hESCs in pluripotency maintaining medium on TCP and CS substrates affects pluripotency and when induced to differentiate whether hESCs favour a specific lineage like hMSCs. We found that despite reducing the substrate stiffness to ten-fold, hESCs remain pluripotent in media with pluripotency factors and differentiation of hESCs occurred only in presence of soluble

signalling molecules. It should be noted that the mESCs are naïve cells while hESCs are primed cells (Ginis *et al*., 2004) and this might affect how cells respond to change in substrate stiffness. The YAP expression in hESCs maintained in pluripotency maintaining medium on TCP and CS substrates remained constant through the stiffness, indicating the YAP expression is not affected by stiffness and helps in maintaining pluripotency.

Next, we explored the effect of differentiation of hESCs cultured on varying stiffness. In certain cell types, such as myotubes (Engler *et al*., 2004; Levy-Mishali *et al*., 2009) and mesenchymal stem cells (Engler *et al*., 2006), optimal differentiation was achieved on a substrate with the same stiffness as the natural microenvironment. Numerous stages of embryogenesis and foetal development are either affected by or generate mechanical forces (Ingber, 2003). More specifically, during gastrulation, blastula epiblast cells ingress (Keller *et al*., 2003; Beloussov and Grabovsky, 2003; Gadue *et al*., 2005), the cells undergo changes in motility and shape, which is attributed to reorganization of the cytoskeleton within the cell (Odell *et al*., 1981; Farge, 2003; Ingber, 2003; Beloussov and Grabovsky, 2003). This change in the cytoskeleton organization is caused by the mechanical forces acting on the cellular surfaces. Therefore, the mechanical forces play an essential role during the lineage-specification of the gastrulation phase.

Our results show that hESCs efficiently differentiated into definitive endoderm lineage upon adding suitable signalling molecules on all the stiffness, TCP and soft substrates alike, as seen by the expression of *SOX17, FOXA2* and *BRACHYURY.* We also checked for the expression of YAP in these differentiating cells, and observed that differentiation has no apparent effect on the YAP expression.

We find that modulating the expression of YAP in differentiating hESCs gives an interesting perspective to the YAP regulation by substrate stiffness. On traditional stiffness (TCP) YAP inhibition has been shown to promote endothelial cell differentiation in hESCs (Quan *et al*., 2021) and also enhanced differentiation of functional stem cell-derived insulin producing beta cells (Rosado-Olivieri *et al*., 2019). When YAP was inhibited by pharmacological inhibitor, TCP showed concurrent result with the literature of decreasing YAP expression during differentiation. Surprisingly, soft substrates showed upregulation of YAP compared to TCP DMSO control. Overexpression of YAP, by supressing Hippo pathway has been shown to promote proliferation of stem cells. Additionally, YAP overexpression

induces transition from primed to the naïve state of hPSCs (Yu *et al*., 2012; Qin *et al*., 2016). Suprisingly, we observed that YAP overepxression favoured hESCs differentiation into endoderm lineage and that YAP expression remains unaffected during the differentiation.

Taken together, our data suggests after a prolonged culture, hESCs remain pluripotent when cultured in pluripotency maintaining medium on soft substrates and differentiate in minimum differentiation inducing medium. This implies that hESCs have different mechanism in sensing the substrate stiffness than hMSCs or mESCs; and for hESCs to differentiate, biochemical signals play a more crucial role than substrate stiffness. During directed differentiation of hESCs on varying stiffness, YAP expression was not effected by the differentiation or by substrate stiffness. This can be varying reasons attributing to these results. It is possible that hESCs respond well to the signalling molecules and are resistant to the substrate stiffness.

Although YAP inhibition and overexpression did not show major changes in YAP expression in soft substrates, but we observed a pattern indicating that YAP inhibition slightly downregulated the differentiation potential whereas YAP overexpression increased the differentiation potential of hESCS into endoderm lineage. Most of the studies using VP and LPA as modulators of YAP have shown YAP effect during the later stages of differentiation in TCP. Our study was focused on understanding the early stages of differentiation, therefore it would be interesting to study the moduations in YAP levels on varying stiffness during late endoderm differentiation. Directly targetting YAP by CRISPR or shRNA would provide further details into the relationship between stiffness and YAP during differentiation. Also, a combinational study with two or more biophysical factors will give us a better understnading on the effect of mechanical forces on the stem cell behvaiour.

Chapter 9: Summary and Conclusion

- ✓ hESCs remain pluripotent when cultured in pluripotency maintaining medium on all stiffness and differentiate in minimum differentiation inducing medium
- ✓ hESCs differentiation does not seem be affected by substrate stiffness
- ✓ Compared to the undifferentiated hESCs and differentiated cells cultured on TCP, no significant change in YAP and pYAP protein levels were seen during endoderm differentiation on soft stiffness
- ✓ YAP expression if affected by the substrate stiffness. On soft stiffness, YAP expression is almost similar in YAP inhibited and overexpressed cells during differentiation.

Chapter 10: Significance of the Study

Our study sheds light into the interplay between the substrate stiffness, hESCs differentiation and YAP regulations in undifferentiated and differentiated hESCs. hPSCs is being actively used in stem cell-based therapies, and regulating hPSCs differentiation will promote better outcome. Our study reveals that more research needs to be conducted in understanding the intrinsic connection between stiffness and hESCs differentiation.

Publications and Poster Presentations

Publications:

1. Manuscript entitled "Impartial effect of substrate stiffness on YAP expression and differentiation in human embryonic stem cells" (under preparation)
2. Virdi, J.K. and Pethe, P., 2022. Soft substrate maintains stemness and pluripotent stem cell-like phenotype of human embryonic stem cells under defined culture conditions. *Cytotechnology, 74(4)*, pp.479-489.
3. Virdi, J.K. and Pethe, P., 2021. Biomaterials regulate mechanosensors YAP/TAZ in stem cell growth and differentiation. *Tissue Engineering and Regenerative Medicine, 18(2),* pp.199-215.
4. Virdi, J.K. and Pethe, P., 2021. Dynamic Interactions Between Stem Cells and Biomaterials. *In Engineering Materials for Stem Cell Regeneration* (pp. 381-398). Springer, Singapore.

Poster presentation:

1. International Society for Stem Cell Research 2021 (ISSCR 2021). 21st – 26th June 2021: Presented poster entitled "Role of Hippo pathway in regulating pluripotency of human embryonic stem cells on soft substrate"
2. Summer Biomechanics, Bioengineering and Biotransport conference 2021 (SB3C2021). 14th – 18th June 2021: Presented poster entitled "Role of substrate stiffness on pluripotency and differentiation of human embryonic stem cells"
3. Advances in Materials Science and Applied Biology (AMSAB). 8th – 10th January 2019. Presented poster entitled "Substrate stiffness regulates YAP/TAZ activity in human pluripotent stem cells."

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References:

Aragona, M., Panciera, T., Manfrin, A., Giulitti, S., Michielin, F., Elvassore, N., Dupont, S. and Piccolo, S., 2013. A mechanical checkpoint controls multicellular growth through YAP/TAZ regulation by actin-processing factors. *Cell*, 154(5), pp.1047-1059.

Beloussov LV, Grabovsky VI. Morphomechanics: goals, basic experiments and models., 2006. *International Journal of Development Biology*, 50(2–3), pp.81–92.

Brusatin G, Panciera T, Gandin A, Citron A, Piccolo S., 2018. Biomaterials and engineered microenvironments to control YAP/TAZ-dependent cell behaviour. *Nature Materials,* 17(12), pp.1063-1075.

Chung H, Lee BK, Uprety N, Shen W, Lee J, Kim J. 2016. Yap1 is dispensable for self-renewal but required for proper differentiation of mouse embryonic stem (ES) cells. *EMBO Reports* 17(4):519–529

Dong C, Beltcheva M, Gontarz P, Zhang B, Popli P, Fischer LA, Khan SA, Park KM, Yoon EJ, Xing X, Kommagani R., 2020. Derivation of trophoblast stem cells from naïve human pluripotent stem cells. *elife*, 9, p.e52504.

Dong J, Feldmann G, Huang J, Wu S, Zhang N, Comerford SA, Gayyed MF, Anders RA, Maitra A, Pan D., 2007. Elucidation of a universal size-control mechanism in Drosophila and mammals. *Cell,* 130(6), pp.1120-1133.

Dumasia, N.P., Khanna, A.P. and Pethe, P.S., 2021. Sonic hedgehog signals hinder the transcriptional network necessary for pancreatic endoderm formation from human embryonic stem cells. *Genes to Cells*, 26(5), pp.282-297.

Dupont, S., Morsut, L., Aragona, M., Enzo, E., Giulitti, S., Cordenonsi, M., Zanconato, F., Le Digabel, J., Forcato, M., Bicciato, S. and Elvassore, N., 2011. Role of YAP/TAZ in mechanotransduction. *Nature*, 474(7350), pp.179-185.

Engler AJ, Griffin MA, Sen S, Bonnemann CG, Sweeney HL, Discher DE., 2004a. Myotubes differentiate optimally on substrates with tissue-like stiffness: pathological implications for soft or stiff microenvironments. *Journal of Cell Biology,* 166(6), pp.877–87.

Engler, A.J., Sen, S., Sweeney, H.L. and Discher, D.E., 2006. Matrix elasticity directs stem cell lineage specification. *Cell*, 126(4), pp.677-689.

Estarás, C., Hsu, H.T., Huang, L. and Jones, K.A., 2017. YAP repression of the WNT3 gene controls hESC differentiation along the cardiac mesoderm lineage. *Genes & Development*, 31(22), pp.2250-2263.

Evans, M.J. and Kaufman, M.H., 1981. Establishment in culture of pluripotential cells from mouse embryos. *Nature*, 292, pp.154-156.

Evans, N.D., Minelli, C., Gentleman, E., LaPointe, V., Patankar, S.N., Kallivretaki, M., Chen, X., Roberts, C.J. and Stevens, M.M., 2009. Substrate stiffness affects early differentiation events in embryonic stem cells. *European Cell Materials*, 18(1), p.e13.

Farge, E., 2003. Mechanical induction of Twist in the Drosophila foregut/stomodeal primordium. *Current Biology*, 13(16), pp.1365-1377.

Gadue, P., Huber, T.L., Nostro, M.C., Kattman, S. and Keller, G.M., 2005. Germ layer induction from embryonic stem cells. *Experimental Hematology*, 33(9), pp.955-964.

Ginis I, Luo Y, Miura T, Thies S, Brandenberger R, Gerecht-Nir S, Amit M, Hoke A, Carpenter MK, Itskovitz-Eldor J, Rao MS., 2004. Differences between human and mouse embryonic stem cells. *Development Biology*, 269(2), pp.360-380.

Hindley, C.J., Condurat, A.L., Menon, V., Thomas, R., Azmitia, L.M., Davis, J.A. and Pruszak, J., 2016. The Hippo pathway member YAP enhances human neural crest cell fate and migration. *Scientific Reports*, 6(1), p.23208.

Holle, A.W., Tang, X., Vijayraghavan, D., Vincent, L.G., Fuhrmann, A., Choi, Y.S., Álamo, J.C. and Engler, A.J., 2013. In situ mechanotransduction via vinculin regulates stem cell differentiation. *Stem Cells*, 31(11), pp.2467-2477.

Hong, J.H., Hwang, E.S., McManus, M.T., AMSTerdam, A., Tian, Y., Kalmukova, R., Mueller, E., Benjamin, T., Spiegelman, B.M., Sharp, P.A. and Hopkins, N., 2005. TAZ, a transcriptional modulator of mesenchymal stem cell differentiation. *Science*, 309(5737), pp.1074-1078.

Huang J, Wu S, Barrera J, Matthews K, Pan D, 2005. The Hippo signaling pathway coordinately regulates cell proliferation and apoptosis by inactivating Yorkie, the Drosophila Homolog of YAP. *Cell* 122(3):421-434.

Ingber, D.E., 2003. Mechanical control of tissue morphogenesis during embryological development. *International Journal of Developmental Biology*, 50(2-3), pp.255-266.

Keller, R., Davidson, L.A. and Shook, D.R., 2003. How we are shaped: the biomechanics of gastrulation. *Differentiation: original article*, 71(3), pp.171-205.

Keung, A.J., Asuri, P., Kumar, S. and Schaffer, D.V., 2012. Soft microenvironments promote the early neurogenic differentiation but not self-renewal of human pluripotent stem cells. *Integrative Biology*, 4(9), pp.1049-1058.

Kumar, A. and Boriek, A.M., 2003. Mechanical stress activates the nuclear factor-kappaB pathway in skeletal muscle fibers: a possible role in Duchenne muscular dystrophy. *The FASEB Journal*, 17(3), pp.386-396.

Lee, S., Stanton, A.E., Tong, X. and Yang, F., 2019. Hydrogels with enhanced protein conjugation efficiency reveal stiffness-induced YAP localization in stem cells depends on biochemical cues. *Biomaterials*, 202, pp.26-34.

Lei, Q.Y., Zhang, H., Zhao, B., Zha, Z.Y., Bai, F., Pei, X.H., Zhao, S., Xiong, Y. and Guan, K.L., 2008. TAZ promotes cell proliferation and epithelial-mesenchymal transition and is inhibited by the hippo pathway. *Molecular and Cellular Biology*, 28(7), pp.2426-2436.

Levy-Mishali, M., Zoldan, J. and Levenberg, S., 2009. Effect of scaffold stiffness on myoblast differentiation. *Tissue Engineering Part A*, 15(4), pp.935-944.

Li, D., Zhou, J., Chowdhury, F., Cheng, J., Wang, N. and Wang, F., 2011. Role of mechanical factors in fate decisions of stem cells. *Regenerative Medicine*, 6(2), pp.229-240.

Lian, I., Kim, J., Okazawa, H., Zhao, J., Zhao, B., Yu, J., Chinnaiyan, A., Israel, M.A., Goldstein, L.S., Abujarour, R. and Ding, S., 2010. The role of YAP transcription coactivator in regulating stem cell self-renewal and differentiation. *Genes & development*, 24(11), pp.1106-1118.

Liu, Y.S. and Lee, O.K., 2014. In search of the pivot point of mechanotransduction: mechanosensing of stem cells. *Cell Transplantation*, 23(1), pp.1-11.

Lorthongpanich, C., Laowtammathron, C., Jiamvoraphong, N., Srisook, P., Chingsuwanrote, P., Klaihmon, P., Damkham, N., Terbto, P., Waeteekul, S., Yaowalak, U. and Issaragrisil, S., 2020. YAP-depleted iPSC MUSIi012-A-2 maintained all normal stem cell characteristics. *Stem Cell Research*, 43, p.101723.

Low, B.C., Pan, C.Q., Shivashankar, G.V., Bershadsky, A., Sudol, M. and Sheetz, M., 2014. YAP/TAZ as mechanosensors and mechanotransducers in regulating organ size and tumor growth. *FEBS Letters*, 588(16), pp.2663-2670.

Makita, R., Uchijima, Y., Nishiyama, K., Amano, T., Chen, Q., Takeuchi, T., Mitani, A., Nagase, T., Yatomi, Y., Aburatani, H. and Nakagawa, O., 2008. Multiple renal cysts, urinary concentration defects, and pulmonary emphysematous changes in mice lacking TAZ. *American Journal of Physiology-Renal Physiology*, 294(3), pp.F542-F553.

Maldonado, M., Luu, R.J., Ico, G., Ospina, A., Myung, D., Shih, H.P. and Nam, J., 2017. Lineage-and developmental stage-specific mechanomodulation of induced pluripotent stem cell differentiation. *Stem Cell Research & Therapy*, 8, pp.1-7.

Maldonado, M., Wong, L.Y., Echeverria, C., Ico, G., Low, K., Fujimoto, T., Johnson, J.K. and Nam, J., 2015. The effects of electrospun substrate-mediated cell colony morphology on the self-renewal of human induced pluripotent stem cells. *Biomaterials*, 50, pp.10-19.

Morgan, J.T., Murphy, C.J., and Russell, P., 2013. What do mechanotransduction, Hippo, Wnt, and TGFβ have in common? YAP and TAZ as key orchestrating molecules in ocular health and disease. *Experimental Eye Research*, 115, pp.1-12.

Morin-Kensicki, E.M., Boone, B.N., Howell, M., Stonebraker, J.R., Teed, J., Alb, J.G., Magnuson, T.R., O'Neal, W. and Milgram, S.L., 2006. Defects in yolk sac vasculogenesis, chorioallantoic fusion, and embryonic axis elongation in mice with targeted disruption of Yap65. *Molecular and Cellular Biology*, 26(1), pp.77-87

Musah, S., Wrighton, P.J., Zaltsman, Y., Zhong, X., Zorn, S., Parlato, M.B., Hsiao, C., Palecek, S.P., Chang, Q., Murphy, W.L. and Kiessling, L.L., 2014. Substratum-induced differentiation of human pluripotent stem cells reveals the coactivator YAP is a potent regulator of neuronal specification. *Proceedings of the National Academy of Sciences*, 111(38), pp.13805-13810.

Odell, G.M., Oster, G., Alberch, P. and Burnside, B., 1981. The mechanical basis of morphogenesis: I. Epithelial folding and invagination. *Developmental Biology*, 85(2), pp.446-462.

Papaspyropoulos, A., Bradley, L., Thapa, A., Leung, C.Y., Toskas, K., Koennig, D., Pefani, D.E., Raso, C., Grou, C., Hamilton, G. and Vlahov, N., 2018. RASSF1A uncouples Wnt from Hippo signalling and promotes YAP mediated differentiation via p73. *Nature Communications*, 9(1), p.424.

Pelham, R.J. and Wang, Y.L., 1997. Cell locomotion and focal adhesions are regulated by substrate flexibility. *Proceedings of the National Academy of Sciences*, 94(25), pp.13661-13665.

Pethe, P., Nagvenkar, P., and Bhartiya, D., 2014. Polycomb group protein expression during differentiation of human embryonic stem cells into pancreatic lineage in vitro. *BMC Cell Biology*, 15(1), pp.18.

Pijuan-Galitó, S., Tamm, C. and Annerén, C., 2014. Serum inter-α-inhibitor activates the Yes tyrosine kinase and YAP/TEAD transcriptional complex in mouse embryonic stem cells. *Journal of Biological Chemistry*, 289(48), pp.33492-33502.

Qin, H., Hejna, M., Liu, Y., Percharde, M., Wossidlo, M., Blouin, L., Durruthy-Durruthy, J., Wong, P., Qi, Z., Yu, J. and Qi, L.S., 2016. YAP induces human naive pluripotency. *Cell Reports*, 14(10), pp.2301-2312.

Quan, Y., Shan, X., Hu, M., Jin, P., Ma, J., Fan, J., Yang, J., Zhang, H., Fan, X., Gong, Y. and Li, M., 2022. YAP inhibition promotes endothelial cell differentiation from pluripotent stem cell through EC master transcription factor FLI1. *Journal of Molecular and Cellular Cardiology*, 163, pp.81-96.

Ramalho-Santos, M., Yoon, S., Matsuzaki, Y., Mulligan, R.C. and Melton, D.A., 2002. " Stemness": transcriptional profiling of embryonic and adult stem cells. *Science*, 298(5593), pp.597-600.

Rosado-Olivieri, E.A., Anderson, K., Kenty, J.H. and Melton, D.A., 2019. YAP inhibition enhances the differentiation of functional stem cell-derived insulin-producing β cells. *Nature Communications*, 10(1), p.1464.

Smith, Q., Chan, X.Y., Carmo, A.M., Trempel, M., Saunders, M. and Gerecht, S., 2017. Compliant substratum guides endothelial commitment from human pluripotent stem cells. *Science Advances*, 3(5), p.e1602883

Takahashi, K. and Yamanaka, S., 2006. Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. *Cell*, 126(4), pp.663-676.

Tian, Y., Kolb, R., Hong, J.H., Carroll, J., Li, D., You, J., Bronson, R., Yaffe, M.B., Zhou, J. and Benjamin, T., 2007. TAZ promotes PC2 degradation through a SCFβ-Trcp E3 ligase complex. *Molecular and Cellular Biology*, 27(18), pp.6383-6395.

Wang, C., Zhu, X., Feng, W., Yu, Y., Jeong, K., Guo, W., Lu, Y. and Mills, G.B., 2016. Verteporfin inhibits YAP function through up-regulating 14-3-3σ sequestering YAP in the cytoplasm. *American Journal of Cancer Research*, 6(1), p.27.

Wu, S., Huang, J., Dong, J. and Pan, D., 2003. hippo encodes a Ste-20 family protein kinase that restricts cell proliferation and promotes apoptosis in conjunction with salvador and warts. *Cell*, 114(4), pp.445-456.

Yu, F.X., Zhao, B., Panupinthu, N., Jewell, J.L., Lian, I., Wang, L.H., Zhao, J., Yuan, H., Tumaneng, K., Li, H. and Fu, X.D., 2012. Regulation of the Hippo-YAP pathway by G-protein-coupled receptor signaling. *Cell*, 150(4), pp.780-791.

Zhao, Y., Fei, X., Guo, J., Zou, G., Pan, W., Zhang, J., Huang, Y., Liu, T. and Cheng, W., 2017. Induction of reprogramming of human amniotic epithelial cells into iPS cells by overexpression of Yap, Oct4, and Sox2 through the activation of the Hippo-Yap pathway. *Experimental and Therapeutic Medicine*, 14(1), pp.199-206.